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gyrA Mutations Associated with Quinolone Resistance in *Bacteroides fragilis* Group Strains

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Mutations in the *gyrA* gene contribute considerably to quinolone resistance in *Escherichia coli*. Mechanisms for quinolone resistance in anaerobic bacteria are less well studied. The *Bacteroides fragilis* group are the anaerobic organisms most frequently isolated from patients with bacteremia and intraabdominal infections. Forty-four clinafloxacin-resistant and-susceptible fecal and clinical isolates of the *B. fragilis* group (eight *Bacteroides fragilis*, three *Bacteroides ovatus*, five *Bacteroides thetaiotaomicron*, six *Bacteroides uniformis*, and 22 *Bacteroides vulgatus*) and six ATCC strains of the *B. fragilis* group were analyzed as follows: (i) determination of susceptibility to ciprofloxacin, levofloxacin, moxifloxacin, and clinafloxacin by the agar dilution method and (ii) sequencing of the *gyrA* quinolone resistance-determining region (QRDR) located between amino acid residues equivalent to Ala-67 through Gln-106 in *E. coli*. Amino acid substitutions were found at hotspots at positions 82 ($n = 15$) and 86 ($n = 8$). Strains with Ser82Leu substitutions ($n = 13$) were highly resistant to all quinolones tested. Mutations in other positions of *gyrA* were also frequently found in quinolone-resistant and -susceptible isolates. Eight clinical strains that lacked mutations in their QRDR were susceptible to at least two of the quinolones tested. Although newer quinolones have good antimicrobial activity against the *B. fragilis* group, quinolone resistance in *B. fragilis* strains can be readily selected in vivo. Mutational events in the QRDR of *gyrA* seem to contribute to quinolone resistance in *Bacteroides* species.

Bacteria belonging to the *Bacteroides fragilis* group are the clinically most important of the anaerobic pathogens and are commonly isolated from intraabdominal infections and other infections below the diaphragm (9, 25). Today, carbapenems, nitroimidazoles, chloramphenicol, and beta-lactam agents in combination with beta-lactamase inhibitors are used for treatment of infections when the *B. fragilis* group is involved. Newly developed quinolones (clinafloxacin, moxifloxacin, levofloxacin, and trovafloxacin) with an extended spectrum of antimicrobial activity compared to earlier quinolones, such as ciprofloxacin, may also be a choice of treatment. However, rates of resistance to these antibiotics are increasing. In an ongoing surveillance study, 16.4% of 1,220 clinical *B. fragilis* group strains from 19 European countries were considered resistant to moxifloxacin, with MICs of ≥4 μg/ml (M Hedberg, Karolinska Institute, Sweden, personal communication).

The quinolones act by interfering with type II topoisomerases, DNA gyrase and topoisomerase IV, which are responsible for cleavage, passage, and rejoining of double-stranded DNA in an ATP-dependent reaction (5). The major difference between the two enzymes is that DNA gyrase has a supercoiling activity, while topoisomerase IV has the ability to decatenate replicated daughter chromosomes. DNA gyrase consists of two subunits, GyrA and GyrB, encoded by the *gyrA* and *gyrB* genes, respectively. Quinolone resistance attributable to *gyrA* mutations was first reported in *Escherichia coli* (12). The *gyrA* quinolone resistance-determining region (QRDR), a highly conserved motif in *E. coli*, is located from amino acid residues Ala-67 through Gln-106. Mutations in this region correlated to quinolone resistance have by now been reported in a number of aerobic bacteria (2, 4, 7, 13, 15, 24, 30). A high level of resistance is commonly associated with substitutions of amino acid residues equivalent to Ser-83 and Asp-87 in *E. coli*. In gram-negative species, quinolone resistance often arises initially from mutations in *gyrA*. If additional mutations in *parC* occur, these usually lead to higher resistance levels (6). Recent data suggest that the *gyrA* gene also plays a role in quinolone resistance in the anaerobe *B. fragilis*. In a study by Onodera et al. (26), the genes encoding the DNA gyrase A and B subunits of *B. fragilis* ATCC 25285 were sequenced. In laboratory mutants of a reference strain resistant to levofloxacin, Ser82 (equivalent to *E. coli* Ser83) of *gyrA* was replaced with Phe, but no mutations were found in *gyrB* (26). Bachoual et al. (1) detected Ser82Phe changes in *gyrA* in 3 of 12 trovafloxacin-resistant (MIC = 4 μg/ml) clinical isolates. This mutation was also found in two of three second-step mutants selected with ciprofloxacin (1). Alterations in GyrA might not be the only explanation for quinolone resistance. Other mechanisms, such as efflux and changes in the outer membrane proteins or alterations in ParC, may also confer quinolone resistance.

Drug efflux has been shown to be an important component in the development of high levels of resistance to this class of agents (8, 10, 17, 27, 31). The presence of an efflux pump in

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B. fragilis, actively pumping out norfloxacin, has been reported by Miyamae et al. (18).

In the present investigation, six American Type Culture Collection (ATCC) strains and 44 clinafloxacin-resistant and -susceptible fecal isolates of the *B. fragilis* group were analyzed as follows: (i) antimicrobial susceptibilities to ciprofloxacin, levofloxacin, moxifloxacin, and clinafloxacin were determined, and (ii) the QRDR of the *gyrA* gene was sequenced.

MATERIALS AND METHODS

Bacterial strains. A total of 44 clinafloxacin-resistant and -susceptible strains of the *B. fragilis* group (eight *B. fragilis*, three *Bacteroides ovatus*, five *Bacteroides thetaiotaomicron*, six *Bacteroides uniformis*, and 22 *Bacteroides vulgatus*) were investigated. All resistant strains derived from a previous study on the impact of clinafloxacin on the intestinal microflora (23). These had been isolated from stool specimens of 12 healthy volunteers treated with clinafloxacin capsules (Parke-Davis Pharmaceutical Research, Berlin, Germany), 200 mg twice a day for 7 days, and had been selected during the administration period or within 2 weeks after withdrawal of the drug using screening plates containing clinafloxacin at 4 μg/ml. The susceptible isolates were derived from stool samples collected prior to clinafloxacin administration or clinical strains from an ongoing European surveillance study (M. Hedberg, Karolinska Institute, Sweden, personal communication). The isolates were identified to genus level by morphology, biochemical tests, and gas-liquid chromatography analysis of metabolic end products (20, 29). Identification to species level was performed phenotypically by fermentation of rhamnose, trehalose, saccharose, and arabinose and biochemical reaction with esculine, indole, and catalase (19, 21). The reference strains *B. fragilis* ATCC 25285, *B. distasonis* ATCC 8503, *B. ovatus* ATCC 8483, *B. thetaiotaomicron* ATCC 29741, *B. uniformis* ATCC 8492, and *B. vulgatus* ATCC 8482 were also included in the study.

Antibiotics. The antimicrobial agents tested were ciprofloxacin and moxifloxacin (Bayer, Leverkusen, Germany), levofloxacin (Roussel Uclaf, Paris, France), and clinafloxacin (Parke Davis Pharmaceutical Research, Ann Arbor, Mich.).

MIC determination. The MICs of ciprofloxacin, levofloxacin, moxifloxacin, and clinafloxacin against the *B. fragilis* group strains were determined by the agar dilution method using brucella agar supplemented with 5% lysed sheep blood according to the published standard (21). The quinolone agents were suspended and diluted according to the manufacturers' instructions. An inoculum of 10^5 CFU per spot was delivered with a Steers replicator. The plates were incubated anaerobically for 48 h at 37°C. The MIC was defined as the lowest concentration of the drug that caused a marked change in the appearance of growth compared to the control plate.

DNA isolation. Chromosomal DNA was extracted from the isolates by suspending fresh colonies in 100 μl of sterile water and boiling for 5 min. After centrifugation for 5 min at 10,000 × *g*, supernatants were collected, and 1:10 dilutions in sterile water were used for PCR.

Amplification of QRDR of *gyrA* gene. PCR amplification of the *gyrA* QRDR of the strains was carried out with primers Pr-BFGBA03 (5′-ATGCTTGAACAA GACAGAATTATAAAG-3′) and Pr-BFGA02 (5′-GACTGTCGCCGTCTACA GAACCG-3′) published by Onodera et al. (26). The primers were purchased from Life Technologies AB, Täby, Sweden. PCRs were carried out in a final volume of 50 μl containing each primer at a concentration of 0.2 pM, 1× PCR buffer, 1.0 mM deoxyribonucleoside triphosphates, 2.0 to 3.0 μM $MgCl_2$, 2.5 U of *Taq* polymerase (Sigma, St. Louis, Mo.), and 10 ng of template. PCR conditions were based on these described previously: 25 cycles of 94°C for 0.5 min (denaturation), 60 to 62°C for 5 min (annealing), and 72°C for 1 min (extension). Optimal conditions varied slightly for different strains and species. PCR products were analyzed by electrophoresis on a 1% agarose gel in TBE buffer (Tris base, boric acid, and EDTA [pH 8.0]). The gel was stained in a 0.5-μg/ml ethidium bromide bath, visualized by UV translumination, and photographed using Polaroid films. DNA fragments 282 to 296 bp long of the QRDR of *gyrA* were obtained.

DNA sequencing and sequence analysis. Free primers and nucleotides from the PCR products were removed by the QIAquick-spin PCR purification kit (Qiagen Inc., Chatsworth, Calif.). Sequencing was carried out using ABI Prism dye terminator cycle sequencing kits with AmpliTaq DNA polymerase, dye terminator chemistry, and an ABI 310 genetic analyzer (Perkin Elmer). Nucleotide and deduced amino acid sequences between the primers were analyzed using the Macintosh DNA program ABI automated DNA sequencer viewer EditView (Perkin Elmer), ClustalW interactive multiple sequence alignment at European Bioinformatics Institute (http://www2.ebi.ac.uk), and the ExPASY molecular biology server at the Swiss Institute of Bioinformatics (Geneva, Switzerland; http://expasy.hcuge.ch). All PCR amplification and sequencing procedures were performed at least twice for each strain. Amino acid sequences of the isolates between the primers were compared between resistant and susceptible isolates and with the corresponding ATCC type strain.

Nucleotide sequence accession numbers. The partial DNA sequences corresponding to the *gyrA* gene of the five ATCC strains of the *B. fragilis* group have been assigned the following GenBank accession numbers: AJ279040 (*B. distasonis* ATCC 8503), AJ279043 (*B. ovatus* ATCC 8488), AJ279039 (*B. thetaiotaomicron* ATCC 2974), AJ 279042 (*B. uniformis* ATCC 8492), and AJ279041 (*B. vulgatus* ATCC 8482).

TABLE 1. Activity of various quinolones against *B. fragilis* group fecal strains

Organism (no. of strains)	MIC range (μg/ml)	MIC_{50}[a] (μg/ml)	No. of resistant strains[b]
B. fragilis (8)			
Ciprofloxacin	2.0–>256	256	7
Levofloxacin	1.0–128	2.0	4
Moxifloxacin	0.5–32	1.0	4
Clinafloxacin	0.125–32	0.25	4
B. ovatus (3)			
Ciprofloxacin	8–256	16	3
Levofloxacin	4–256	8	3
Moxifloxacin	1.0–2.0	0	0
Clinafloxacin	0.25–2.0	0.5	0
B. thetaiotaomicron (5)			
Ciprofloxacin	8–256	64	5
Levofloxacin	2.0–256	8.0	4
Moxifloxacin	0.5–32	4.0	3
Clinafloxacin	1.0–8.0	1.0	2
B. uniformis (6)			
Ciprofloxacin	32–256	256	6
Levofloxacin	16–128	64	6
Moxifloxacin	2.0–64	32	5
Clinafloxacin	0.25–32	8.0	5
B. vulgatus (22)			
Ciprofloxacin	8–>256	256	22
Levofloxacin	2.0–256	128	20
Moxifloxacin	1.0–128	32	20
Clinafloxacin	0.125–32	8.0	20
All (44)			
Ciprofloxacin	2.0–>256	256	43
Levofloxacin	2.0–256	128	37
Moxifloxacin	0.25–128	16	32
Clinafloxacin	0.125–32	8.0	31

[a] MIC_{50}, MIC for 50% of strains.
[b] MIC values of ≥4 μg/ml are considered as indicating resistance; see text.

RESULTS

The MIC values of the various quinolones are listed in Table 1. Clinafloxacin was the most active of all the quinolones, followed by moxifloxacin, levofloxacin, and ciprofloxacin. Thirty-one isolates were classified as resistant to all four quinolones. In the absence of breakpoint criteria for the quinolones tested, the recommended breakpoint concentration for trovafloxacin, ≥4 μg/ml, was used (22). In general, strains resistant to moxifloxacin and clinafloxacin usually had higher MIC values (≥32 μg/ml) to ciprofloxacin and levofloxacin than the moxifloxacin- and clinafloxacin-susceptible strains.

The sequenced fragment of the *gyrA* QRDR in *B. fragilis*

TABLE 2. Amino acid substitutions[a] in QRDR of GyrA in *B. fragilis* group strains compared to ATCC strains, and MIC of four quinolones

Species	Type strain or no. of isolates	Amino acid at position:														MIC (μg/ml)			
		30	40	49	53	61	68	80	82	83	86	88	94	100	101	Ciprofloxacin	Levofloxacin	Moxifloxacin	Clinafloxacin
B. fragilis	ATCC 25285	S	F	Y	E	P	I	G	S	S	F	M	E	P	L	8.0	0.5	0.125	0.125
	3	S	F	Y	E	P	I	G	S	S	F	M	E	P	L	2–>256	1–2	0.125–0.5	0.125–0.25
	1	A	F	Y	E	A	I	G	S	S	G	L	D	P	L	4	2	1	0.25
	4	A	L	Y	E	A	I	G	L	S	G	L	D	P	L	256	128	16–32	8–32
B. distasonis	ATCC 8503	S	F	F	E	P	I	G	S	S	F	M	T	P	L	8	8	0.25	0.125
B. ovatus	ATCC 8483	S	F	Y	E	P	I	G	S	S	F	M	E	P	L	16	8	0.5	0.25
	1	S	F	Y	E	P	I	G	S	S	Y	M	E	P	L	8	4	1	0.25
	1	S	F	Y	E	P	I	G	C	S	F	M	E	P	L	16	8	2	0.5
	1	S	F	Y	E	P	I	G	F	S	F	M	E	P	L	256	256	2	2
B. thetaiotaomicron	ATCC 29741	S	F	Y	E	P	I	G	S	S	Y	M	E	P	L	16	8	0.5	0.25
	3	S	F	Y	E	P	I	G	S	S	Y	M	E	P	L	8–64	2–8	1–4	0.25–1.0
	2	S	F	Y	E	P	I	G	S	S	F	M	E	P	L	256	128–256	32	8
B. uniformis	ATCC 8492	S	F	F	G	P	V	G	S	S	G	L	P	M	L	8	8	1	0.5
	1	S	F	F	G	P	V	G	S	S	G	L	P	M	L	32	16	2	0.5
	5	S	F	F	G	P	V	G	L	S	G	L	P	M	L	32–256	64–128	16–64	8–32
	1	A	F	Y	E	A	I	G	S	S	G	L	D	P	L	8	2	1	0.125
B. vulgatus	ATCC 8482	A	F	Y	E	A	I	G	S	S	G	L	D	P	L	16	2	1	0.125
	14	S	F	F	G	P	V	G	S	S	G	L	P	M	V	256–>256	2–>256	2–32	0.125–16
	2	S	F	F	G	P	V	D	S	S	G	L	P	M	V	256–>256	128–256	32–128	8–32
	1	S	F	F	G	P	V	G	S	S	G	L	P	M	L	256	256	32	32
	1	S	F	Y	G	P	V	G	L	S	G	L	P	M	L	>256	64	64	8
	2	S	F	F	G	P	V	G	L	S	G	L	P	M	L	>256–256	64–128	16–32	8–16
	1	S	F	F	G	P	V	G	L	S	G	L	P	M	V	256	256	32	16

[a] Only indicated when differences from type strain are abundant.

ATCC 2528 was identical in nucleotide and amino acid sequences to that previously assigned in GenBank (accession no. AB017712) and had 72% identity with *E. coli*. The pairwise scores among the study ATCC strains regarding amino acid sequences matched with *B. fragilis* ATCC 25285 and *E. coli* as follows: *B. distasonis* ATCC 8503, 95 and 73%; *B. ovatus* ATCC 8483, 100 and 72%; *B. thetaiotaomicron* ATCC 2974, 98 and 72%; *B. uniformis* ATCC 8492, 89 and 72%; and *B. vulgatus* ATCC 8482, 92 and 72%, respectively. It was also observed among the clinical isolates that the partial *gyrA* sequences of almost all *B. vulgatus* isolates had higher similarity with *B. uniformis* ATCC 8492 than with *B. vulgatus* ATCC 8482. Mutations in the *gyrA* QRDR are listed in Table 2. At resistance hotspots 82 (equivalent to Ser-83 in *E. coli*) and 86 (equivalent to Asp-87 in *E. coli*), amino acid exchanges were found in 15 and 8 strains, respectively. Thirteen isolates (four *B. fragilis*, five *B. uniformis*, and four *B. vulgatus*) had Ser82Leu changes, while in two *B. ovatus* strains Ser82 was replaced with Phe or Cys. The strains with Ser82Leu mutations were resistant to all four quinolones tested, while those with Phe or Cys substitutions of Ser82 were susceptible to at least two of the quinolones. Eight isolates (five *B. fragilis*, one *B. ovatus*, and two *B. thetaiotaomicron*) carried amino acid exchanges at position 86. Two *B. thetaiotaomicron* isolates with a single mutation of Tyr86 to Phe were resistant to all four quinolones, while one *B. ovatus* isolates with a single mutation of Phe86 to Tyr showed a susceptibility pattern similar to that of the type strain. Phe86Gly substitutions were present in five strains, of which four with additional Ser82Leu mutations were resistant to all quinolones tested. Eight isolates, three *B. fragilis*, three *B. thetaiotaomicron*, one *B. uniformis*, and one *B. vulgatus*, lacked mutations and were also susceptible to at least two of the quinolones tested. The remaining 36 fecal isolates, of which 31 were resistant to all quinolones tested, had one to nine mutations in their QRDRs. Other more unusual amino acid substitutions occurred most frequently at positions 30, 49, 53, 61, 68, 94, and 100 and could not be clearly related to resistance.

DISCUSSION

The similarities of the partial QRDR sequences of the ATCC strains with *B. fragilis* ATCC 25285 varied between 89 and 100%. Ser82 was conserved in all studied *B. fragilis* group reference strains, but at position 86 Phe, Gly, or Tyr was present. These substitutions for the Asp (in *E. coli*) may help to explain why *B. fragilis* group strains are intrinsically resistant to many of the quinolones, since these mutations are common in quinolone-resistant *E. coli* strains. The partial *gyrA* sequences of almost all clinical *B. vulgatus* isolates had higher similarity with *B. uniformis* ATCC 8492 than with *B. vulgatus* ATCC 8482. ATCC strains for specific species might not necessarily represent a "wild-type" sequence for that species. Some variability found in clinical isolates may be due to incomplete genetic information about the range of mutations in clinical strains. A mosaic of amino acid changes might be found among a large database for each *Bacteroides* spp.

The possibility of misidentification in the present investigation is low, since phenotyping results were clear and identical on repeated testing. The routine phenotypic identification has also been shown to agree with the more accurate genotyping by restriction fragment length polymorphism analysis of amplified 16S rDNA (28).

Strains with Ser82Leu mutations were resistant to all quinolones tested, while strains with other Ser82 substituents than Leu were susceptible to two or more of the quinolones tested. In two previous studies on quinolone-resistant *B. fragilis* strains, Ser82 was replaced with Phe (1, 23). The MIC values of the in vivo-selected isolates in our study were considerably higher than those reported by Bachoual et al. (1) and Onodera et al. (26). Mutations at hotspot position 86 were found in eight isolates (five *B. fragilis*, one *B. ovatus*, and two *B. thetaiotaomicron*). Five of these showed resistance to all quinolones tested, of which two carried single point mutations of Tyr86 to Phe. One strain with a single mutation of Phe86 to Tyr was susceptible to the two most active quinolones. Four of the five isolates in which Phe86 was replaced with Gly were resistant to all quinolones tested, although these had additional Ser82Leu mutations. In contrast, one isolate with the Phe86Gly substitution but no Ser82Leu replacement showed quinolone susceptibility similar to that of the type strain. These results suggest that replacement at codon 86 with Phe might contribute to high quinolone resistance, while substitutions with Tyr or Gly do not. In a similar way, isolates with Leu replacing Ser at position 82 were resistant to moxifloxacin and clinafloxacin, while those with Phe or Cys substitutions at the same position were not. It has previously been shown in *E. coli* that different amino acid substitutions in the same position in GyrA can produce different levels of resistance (11). Among some strains with identical GyrA sequences, a wide range of MIC values could be noted, indicating involvement of other resistance mechanisms. In the present study, resistant isolates had been selected using only clinafloxacin, and the correlation between mutations and MIC values for other agents might be inconsequent, since each agent may select for different mutations. However, all clinafloxacin-resistant isolates also showed resistance to moxifloxacin, levofloxacin, and ciprofloxacin. The resistance pattern among the isolates was consistent; no remarkable differences were shown in the order of activity between the different quinolones.

Newer quinolones like moxifloxacin and clinafloxacin could be potential agents against infections caused by the *B. fragilis* group since they have excellent in vitro activity against anaerobes. Comparative studies have shown that clinafloxacin has the highest activity among the quinolones (3, 14, 16). A possible disadvantage is that *B. fragilis* group strains resistant to quinolones can readily be selected in the intestinal microflora. In a previous study by our group, it has been shown that a shift from susceptible to resistant intestinal strains occurred in 9 of 12 subjects during clinafloxacin treatment (23). Thus, it is important to restrict the quinolone class of antibiotics to appropriate indications, due to the risk of emergence of resistance.

In conclusion, strains of the *B. fragilis* group with reduced susceptibility to quinolones harbored a number of amino acid substitutions, including mutations at hotspot positions in their QRDRs of GyrA. Fifteen of 31 strains resistant to all quinolones tested had mutations at hotspot positions, mainly Ser82Leu. Sixteen highly resistant strains lacked hotspot mutations, indicating the involvement of other mechanisms of resistance. Further studies are needed to increase the understanding on quinolone resistance in *Bacteroides* species.

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